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Some differences in the properties of carnitine palmitoyltransferase activities of the mitochondrial outer and inner membranes

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Recent evidence has shown that the outer, overt, malonyl-CoA-inhibitable carnitine palmitoyltransferase (CPT_o) activity resides in the mitochondrial outer membrane [Murthy & Pande (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 378–382]. A comparison of CPT_o activity of rat liver mitochondria with the inner, initially latent, carnitine palmitoyltransferase (CPT_i) of the mitochondrial inner membrane has revealed that the presence of digitonin and several other detergents inactivates CPT_o activity. The CPT_i activity, in contrast, was markedly stimulated by various detergents and phospholipid liposomes. These findings explain why in previous studies, which used digitonin or other detergents to expose, separate and purify the CPT activities, the inferences were drawn that (*a*) the ratio of latent to overt CPT was quite high, (*b*) both the CPT activities could be ascribed to one active protein recovered, and (*c*) the observed lack of malonyl-CoA inhibition indicated possible loss/separation of a putative malonyl-CoA-inhibition-conferring protein. Although both CPT_o and CPT_i were found to catalyse the forward and the backward reactions, CPT_o showed greater capacity for the forward reaction and CPT_i for the backward reaction. The easily solubilizable CPT, released on sonication of mitoplasts or of intact mitochondria under hypo-osmotic conditions, resembled CPT_i in its properties. When octyl glucoside was used under appropriate conditions, 40–50% of the CPT_o of outer membranes became solubilized, but it showed limited stability and decreased malonyl-CoA sensitivity. Malonyl-CoA-inhibitability of CPT_o was decreased also on exposure of outer membranes to phospholipase C. When outer membranes that had been exposed to octyl glucoside or to phospholipase C were subjected to a reconstitution procedure using asolectin liposomes, the malonyl-CoA-inhibitability of CPT_o was restored. A role of phospholipids in the malonyl-CoA sensitivity of CPT_o is thus indicated.

INTRODUCTION

Functional studies have established that the carnitine-dependent oxidation of fatty-acyl groups requires the involvement of two differentially localized carnitine palmitoyltransferase (CPT) activities of mitochondria. In intact mitochondria, CPT is known to exist in an overt (CPT_o) and a latent (CPT_i) form. The latter is exposed by membrane-disruptive agents/procedures, and it seems to reside on the inner side of the inner membrane (Bremer, 1983; Bieber & Farrell, 1983; Hoppel & Brady, 1985). The CPT_o activity was previously assigned a localization on the outer side of the inner membrane, but our subsequent evidence has shown that it resides on the inner side of the outer membrane (Pande & Murthy, 1986; Murthy & Pande, 1987). In their environment *in situ*, these two activities show appreciable differences in their sensitivity to certain inhibitors and in kinetic and regulatory properties. The question of whether these two CPT activities reside in the same protein molecule that expresses disparate properties owing to differences in their membrane environment, or in two proteins, has remained unresolved, as evidence supporting both has been described (Miyazawa *et al.*, 1983; Bieber & Farrell, 1983; Fiol & Bieber, 1984; Kiorpes *et al.*, 1984; Declercq *et al.*, 1985). In those studies separation and/or purification of the CPT activity(ies) was carried out with detergents, and the possibility that the results were influenced by the use of detergents was not eliminated. We have compared some of the properties of the CPT_o and CPT_i activities using outer-membrane vesicles (OMV) and inner-membrane vesicles (IMV), isolated without using detergents, as sources of the two CPTs respectively. We report here that under these conditions markedly different properties have emerged for the two membrane-bound forms of CPT activities. An examination of the effects of digitonin and other detergents has revealed that their use in previous studies introduced artifacts, lack of recognition of which provided support to the deductions that both the CPT activities reside in the same protein (Bergstrom & Reitz, 1980; Clarke & Bieber, 1981*a*,*b*; Miyazawa *et al.*, 1983). The possibility that the CPT_o and CPT_i may be different proteins therefore needs to be reconsidered. An abstract of some of this work has appeared (Pande & Murthy, 1987). Some very similar observations and conclusions have been described by Lund (1987) in a paper that appeared after the submission of our manuscript.

Abbreviations used: CPT, carnitine palmitoyltransferase; OMV, outer-membrane vesicles; IMV, inner-membrane vesicles; CPT_o, overt CPT of mitochondrial outer membrane; CPT_i, inner-membrane (initially latent) CPT.

MATERIALS AND METHODS

Materials

[1-^{14}C]Palmitoyl-CoA was from Amersham. Digitonin was purchased from Sigma and recrystallized before use as recommended (Kun *et al.*, 1979).

Methods

Rat liver mitochondria were isolated as described by Parvin & Pande (1979). Preparation of mitochondrial outer membranes by the procedure of Parsons *et al.* (1966) was described in Murthy & Pande (1987). To obtain IMV, the liver mitochondrial mitoplasts were prepared by the removal of outer membrane by swelling mitochondria in 20 mM-potassium phosphate (pH 7.2)+0.02% bovine serum albumin (Murthy & Pande, 1987) and then were processed as described by Coty *et al.* (1979). Briefly, the mitoplasts were suspended in water (2 mg/ml) and the 'ghosts' were pelleted by centrifuging at 10000 *g* for 15 min. These 'ghosts' were suspended at 10 mg/ml in 210 mM-mannitol/70 mM-sucrose/10 mM-Tris/HCl (pH 7.4/0.5 mM-EDTA and sonicated for 8 × 15 s, with a 15 s cooling period between each at 0 °C. The sonicated suspension was centrifuged at 10000 *g* for 10 min to remove unbroken mitoplasts. The supernatant was then centrifuged at 110000 *g* for 1 h to obtain IMV as a sediment, and a 'supernatant' fraction with the soluble CPT. IMV thus obtained had $\leqslant 5\%$ outer-membrane contamination as judged from the associated monoamine oxidase activity, and cytochrome *c* oxidase was enriched by nearly 4-fold. The 'supernatant' fraction had $\leqslant 2\%$ of the cytochrome *c* oxidase activity of mitochondria.

Carnitine palmitoyltransferase assay

Assay of CPT in the forward direction (palmitoyl-CoA to palmitoylcarnitine) was by the method of Saggerson & Carpenter (1982) as described in Murthy & Pande (1987), except that 2 mM-carnitine was used. Briefly, the assay system at 30 °C, in 250 μl final volume, contained 220 mM-sucrose, 40 mM-KCl, 10 mM-Tris/HCl, pH 7.4, 1 mM-EGTA, 1 mM-dithiothreitol, 1.3 mg of fatty-acid-free bovine serum albumin/ml, the indicated concentrations of [1-^{14}C]palmitoyl-CoA and 2 mM-carnitine. CPT in the backward direction (palmitoylcarnitine to palmitoyl-CoA) was assayed as described in Pande *et al.* (1986). In this case, the assay system (final volume 100 μl) at 30 °C, contained 50 mM-triethanolamine/HCl, pH 7.4, 100 mM-KCl, 6 mM-CoASH, 15 mM-dithiothreitol and 200 μM-palmitoyl[^{3}H]carnitine.

Phospholipase treatment of outer- and inner-membrane vesicles of liver mitochondria

OMV or IMV (0.07–0.1 mg of protein) were exposed to 5 units of salt-depleted phospholipase A_2 (from pig pancreas) in 0.2 ml of a medium containing 50 mM-Hepes, pH 7.4, and 3 mM-$CaCl_2$ for 15 min at 37 °C. The tubes were then kept on ice and received 25 μl of 900 mM-potassium phosphate (pH 7.4)/45 mM-EDTA/9 mM-dithiothreitol; samples were removed for CPT assay. For phospholipase C treatment, OMV or IMV (about 0.2 mg of protein) were incubated, in 0.1 ml of 50 mM-Hepes/KOH, pH 7.4, without or with indicated quantities of phospholipase C, for 15 min at 37 °C. The tubes were then kept on ice, and 0.2 ml of 7.5 mM-EDTA/1.5 mM-dithiothreitol/150 mM-potassium phosphate, pH 7.4, was added and the tubes were centrifuged at 100000 *g* for 30 min. The pellets were suspended in 100 mM-potassium phosphate (pH 7.4)/1 mM-EDTA/1 mM-dithiothreitol and the CPT activity was monitored.

Preparation of liposomes with either asolectin or purified phospholipids was as described by Noël *et al.* (1985).

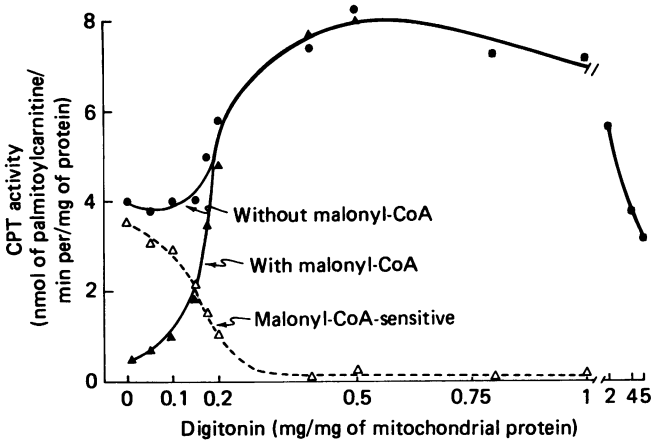

Fig. 1. Effect of digitonin on liver mitochondrial CPT activity and its inhibition by malonyl-CoA

Freshly prepared mitochondria (0.75 mg of protein) were incubated at 0 °C for 5 min in a final volume of 30 μl in a medium containing 150 mM-KCl, 5 mM-Tris/HCl, pH 7.4, 1 mM-EGTA and indicated concentrations of digitonin. After the incubation, the mitochondrial protein concentration was lowered to 2.5 mg/ml by the addition of KCl/Tris/HCl/EGTA, and samples were rapidly removed for CPT assay at 30 °C. CPT was assayed with 50 μM-palmitoyl-CoA, with or without 200 μM-malonyl-CoA. Closely overlapping values with and without malonyl-CoA were obtained for 0.75 mg of digitonin/mg of protein. Other details were as in the Materials and methods section. The malonyl-CoA-sensitive activity represents the difference of the activity measured without and with malonyl-CoA. Results are means from three or four separate experiments.

RESULTS AND DISCUSSION

Effect of digitonin and detergents on CPT activities

These agents have been used for the separation and/or solubilization of the two CPT activities (Bergstrom & Reitz, 1980; Bieber & Fiol, 1986), but, during the course of the present work, we found several of them unsuitable for CPT_o. Thus the CPT activity released on exposing mitochondria to low concentrations of digitonin has been taken to be that derived from the outer CPT (Hoppel & Tomec, 1972; Bergstrom & Reitz, 1980). Subsequent work by others (Declercq *et al.*, 1985) has indicated, however, that digitonin may not be selective for the solubilization of outer CPT (see the discussion in Murthy & Pande, 1987). We have now found that digitonin inactivates the CPT_o, and the activity which it exposes is derived from CPT_i and the initially latent easily solubilizable CPT (see below). This conclusion is based on the following observations: (*a*) the malonyl-CoA-sensitive CPT activity of mitochondria was very sensitive to inhibition by digitonin whether measured in

Table 1. Effect of various detergents on CPT activities of various mitochondrial fractions

In a medium containing 100 mM-potassium phosphate, pH 7.4, 20% glycerol, 1 mM-EDTA and 1 mM-dithiothreitol, fractions of OMV, IMV, or the supernatant obtained after centrifuging the sonicated mitoplast ghosts at 110000 *g* for 1 h were incubated with various detergents for 45 min at 0 °C, except for digitonin. The ratios of detergent to protein during this 0 °C incubation were as indicated below. Details of the digitonin treatment were as in the legend for Fig. 1. CPT activity was measured with 50 μM-palmitoyl-CoA and 10–20 μg protein of OMV or IMV or 30 μg of supernatant protein (see the Materials and methods section). Values are means ± S.E.M. for four to six observations: $*P <$ or $\ll 0.05$ compared with 'None'.

Detergent	Detergent:protein ratio (w/w)	CPT activity (nmol/min per mg of protein)		
		OMV	IMV	Supernatant
None	–	110±4.5	21±1.4	8±0.4
15 mM-Octyl glucoside	10:1	69±5.1*	34±1.5*	25±0.8*
30 mM-Octyl glucoside	10:1	55±4.6*	45±3.2*	23±1.2*
40 mM-Octyl glucoside	10:1	44±2.3*	43±2.4*	26±2.1*
1% Triton X-100	1:1	34±1.2*	32±3.0*	11±0.7*
0.5% Tween-20	5:1	112±5.0	45±0.9*	26±1.0*
2% Tween-20	20:1	44±2.7*	53±2.1*	26±1.2*
5% Tween-20	50:1	25±1.1*	70±3.2*	24±2.1*
15 mM-Cholate	10:1	73±4.1*	38±1.2*	10±0.2*
10 mM-CHAPS	5:1	57±3.7*	29±1.3*	12±0.8*
0.2% Digitonin	2:1	49±1.5*	17±0.8	8±0.2
0.5% Digitonin	5:1	5±0.4*	14±1.0*	4±0.1*
1.6% Digitonin	25:1	0	8±1.5*	3±0.1*

intact mitochondria (Fig. 1, △) or in OMV (Table 1); (*b*) although the digitonin inhibition of malonyl-CoA-sensitive CPT activity accompanied the reciprocal increase in malonyl-CoA-insensitive CPT activity (compare curves △ with ▲ in Fig. 1), this did not result from the conversion of malonyl-CoA-sensitive CPT into the insensitive form, because the digitonin inhibition of the CPT_o activity [Table 1; the data of Declercq *et al.* (1985) also lend themselves to this interpretation] of isolated OMV did not accompany any change in the malonyl-CoA-inhibitability of the surviving CPT activity as ⩾ 90% of it was still inhibited by 200 μM-malonyl-CoA.

Detergents have been used to solubilize and to purify CPT, but the fact that the CPT_o activity is lost under such conditions and that the activity of the CPT_i is concurrently stimulated, as the data of Table 1 show, has not been recognized previously. Digitonin was the only detergent among those examined that did not stimulate the CPT_i activity of isolated IMV but inhibited it instead. It should be noted from Table 1 that, although 2 mg of digitonin/mg of OMV protein caused nearly 50% inhibition of CPT_o activity, a corresponding inhibition of CPT_i activity required over 10-fold as much digitonin. With intact mitochondria (Fig. 1), a lower ratio of digitonin to protein proved inhibitory than with OMV (Table 1). With intact mitochondria, however, the ratio of mg of digitonin to mg of membrane surface proteins that do actually become exposed to digitonin would be much greater when related to those proteins only rather than to the total mitochondrial protein, because, in intact mitochondria, initially digitonin would remain inaccessible to many proteins of the inner membrane and to all of the matrix proteins. The interpretation in previous studies (Bergseth *et al.*, 1986) that digitonin (and other detergents) abolishes malonyl-CoA inhibition because of the dissociation of a putative

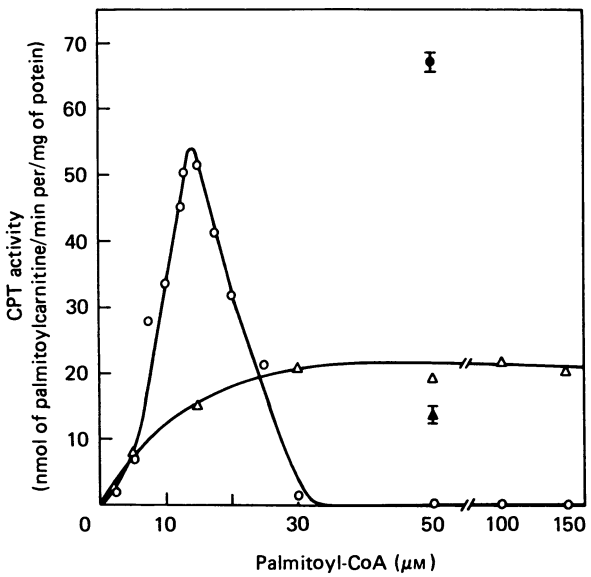

Fig. 2. Effect of palmitoyl-CoA concentration on CPT activity of mitochondrial membranes

The incubation system in 125 μl contained 100 mM-potassium phosphate (pH 7.4), 1 mM-EDTA, 1 mM-dithiothreitol, 2 mM-[^{14}C]carnitine (0.35 μCi) and palmitoyl-CoA as shown. Assays were started by the addition of 2.5 μg of protein of OMV (○) or IMV (△). Incubations were for 5 min at 30 °C. Formation of [^{14}C]-palmitoylcarnitine was monitored by the butanol extraction procedure as described by Pande & Blanchaer (1970). Results are averages of three observations. At 50 μM-palmitoyl-CoA the CPT activity was measured also in the presence of 165 μg of bovine serum albumin; these values (means ± S.E.M., $n = 4$) with OMV and IMV are shown as ● and ▲ respectively.

Table 2. Comparison of the forward and the backward activities of CPT associated with the various mitochondrial fractions

OMV, IMV and supernatant fraction of mitochondria were prepared as described in the Materials and methods section. CPT was assayed in the forward direction with 50 μM-[^{14}C]palmitoyl-CoA and 2 mM-carnitine and in the backward direction with 200 μM-palmitoyl[^{3}H]carnitine and 6 mM-CoASH. Results are means ± S.E.M. for three to six observations.

Mitochondrial fraction	CPT activity (nmol/min per mg of protein)		Backward/forward ratio
	Forward direction	Backward direction	
OMV	96±4.6	36±2.5	0.38
IMV	18±1.1	186±4.3	10.3
Supernatant	7±0.5	66±2.1	9.4

malonyl-CoA-inhibition-conferring protein therefore needs amendment.

The CPT activity of mitochondrial extracts and of the enzyme purified by using Triton X-100, and hence representing CPT_i, in view of the present finding, is known to be inhibited by palmitoyl-CoA (Pande *et al.*, 1986). Our present data have revealed, however, that the CPT activity of OMV is remarkably more sensitive to this inhibition than is the CPT of IMV (Fig. 2). The palmitoyl-CoA inhibition of CPT_o involved a detergent-type inactivation, insofar as it was not reversed by the subsequent lowering of palmitoyl-CoA concentration or addition of bovine serum albumin. Under the conditions of these experiments, with 50 μM-palmitoyl-CoA, addition of bovine serum albumin before the CPT was essential for demonstrating CPT activity with OMV, but not with IMV (Fig. 2; compare the filled with the empty symbols).

Inclusion of 200 μg of phospholipid liposomes (phosphatidylcholine/phosphatidylethanolamine/phosphatidylinositol, 4:2:1, by wt.) in the assay system led to a 4-fold increase in the activity of CPT_i, but had no effect on the CPT_o activity. Under the conditions described in the Materials and methods section, exposure of the OMV and the IMV to phospholipase A_2 led to 93% loss of CPT_o activity, but only 34% loss of CPT_i activity. On exposure to 100 units of phospholipase C/mg of protein, nearly 85% of both activities was lost. In none of these was the lost activity restored on inclusion of the phospholipid liposomes in the assay medium.

Relative abilities of CPT_o and CPT_i to catalyse the forward and the backward reactions

The question of whether the outer and the inner CPTs both catalyse the forward and the backward reactions has been of interest ever since Kopec & Fritz (1971, 1973) reported that the inner CPT seemed unable to catalyse the forward reaction, and it is on this basis that some of the data on CPT deficiency have been interpreted (Hostetler *et al.*, 1978). Functional studies with intact mitochondria have, however, indicated that the inner CPT does convert acyl-CoAs into acylcarnitines (Brass & Hoppel, 1980; Moore *et al.*, 1982). Our data in Table 2 show that, whereas both CPTs catalysed both the reactions, their relative capacities for the backward to forward reaction when measured at near-saturating concentrations of substrates varied markedly; thus CPT_o showed greater capacity for the forward reaction and CPT_i for the backward reaction. Under the conditions of these experiments (Table 2), the substrate concentrations employed were near saturating inasmuch as the activity was not increased noticeably by a further doubling in substrate concentrations, palmitoyl-CoA and carnitine for the forward reaction and palmitoylcarnitine and CoA for the reverse reaction. Although detailed kinetic studies remain to be performed, these data obtained for the membrane-bound forms of CPTs indicate that the properties of these two carnitine long-chain acyltransferases are poised to favour their major function of delivering fatty acyl groups to the site of the mitochondrial β-oxidation.

The readily releasable CPT of mitochondria resembles CPT_i activity

Although it has generally been believed that the mitochondrial CPTs are membrane-associated enzymes, it has also been noted that a part of the CPT activity is readily released in the supernatant by subjecting mitochondria to procedures such as hypo-osmotic swelling, freeze–thaw and sonication. The activity so released has

Table 3. Effects of malonyl-CoA and 2-tetradecylglycidyl-CoA on the CPT activities of OMV, IMV and the supernatant fractions

CPT was assayed with 20 μM-palmitoyl-CoA; other details were as in the Materials and methods section. Results are means ± S.E.M. for four observations (N.D., not determined): *$P \ll 0.01$ compared with the no-addition controls.

Addition	Concn. (μM)	CPT activity (nmol/min per mg ofprotein)		
		OMV	IMV	Supernatant
None	–	46.7±2.4	15.1±1.2	8.0±0.4
Malonyl-CoA	0.1	24.3±1.5*	N.D.	N.D.
	1.0	4.0±0.1*	13.5±1.6	7.8±0.3
	200.0	2.3±0.1*	13.1±0.9	6.5±0.5
2-Tetradecylglycidyl-CoA	0.15	25.7±2.0*	N.D.	7.0±0.7
	1.0	3.3±0.5*	12.1±0.7	6.9±0.7
	20.0	0	8.2±0.7*	5.5±0.4*

been considered as derived from the outer CPT (West *et al.*, 1971; Hoppel *et al.*, 1986; Derrick *et al.*, 1986). In our experiments the CPT_o activity associated with the isolated OMV could account for the entire overt CPT activity of intact mitochondria (Pande & Murthy, 1987; Murthy & Pande, 1987), showing that, during the isolation of OMV, the CPT_o activity was not being released. In line with this, we found that the CPT_o activity associated with the isolated OMV was quite resistant to solubilization; it could not be readily solubilized by sonication, salt extractions, phospholipase digestion, or by several anionic, cationic and non-ionic detergents tested under a variety of conditions. An exception was octyl glucoside. With 40 mM-octyl glucoside, 40–50% of the initial malonyl-CoA-sensitive activity was recovered in the 110000 $g \times 60$ min supernatant fraction. The solubilized activity, however, proved unstable, as it was completely lost within 24 h at 0 °C. A comparison of the CPT activity released by the sonication of mitoplast ghosts showed that it closely resembled CPT_i in the following properties: (*a*) relative insensitivity to inhibition by malonyl-CoA, tetradecylglycidyl-CoA (Table 3) and digitonin (Table 1), (*b*) detergent enhancement of activity (Table 1), and (*c*) higher capacity for the backward than for the forward reaction (Table 2). Hypo-osmotic swelling of intact liver mitochondria (5–10 mg/ml) in 10 mM-potassium phosphate (pH 7.2)/(1 mM-EDTA/1 mM-dithiothreitol, followed by sonication, also led to the release of about 15% of the total CPT activity into the medium, which was not sedimented by centrifugation at 110000 *g* for 60 min. This soluble CPT resembled the CPT released by the sonication of mitoplast ghosts in the various properties mentioned above, including complete lack of sensitivity to inhibition by malonyl-CoA and 2-tetradecylglycidyl-CoA (results not shown).

Because it is known that conventionally isolated mitochondria have some peroxisomal contamination, that the peroxisomal carnitine octanoyltransferase shows some activity with palmitoyl-CoA and palmitoylcarnitine, and that this activity is readily released into the supernatant by freezing and thawing, swelling and sonication (Farrell *et al.*, 1984), the possibility that the easily solubilizable CPT associated with the mitochondrial fraction is of peroxisomal origin, in part, cannot be eliminated at present.

Proportions of CPT_o and CPT_i activities in liver mitochondria

The estimates of the relative proportions of the outer and the inner CPT activities of liver mitochondria from normal fed rats have ranged from 1:1 to 1:$\geqslant$10 (for review, see Bieber & Farrell, 1983; Lund & Bremer, 1983). The higher ratios have been obtained in experiments that have utilized digitonin or other detergents; our present data indicate that digitonin and other detergents have given high rates because they inhibit CPT_o while exposing and/or activating the CPT_i activity. Our assessments indicate a ratio of 1:1.5–2 for the overt to the latent activities when measured in the forward direction with near-saturating concentrations of palmitoyl-CoA (50 μM) and carnitine (2 mM). This is based on the following considerations. (*a*) Fig. 1 shows that the specific activity of the initially overt, malonyl-CoA-sensitive, CPT_o activity of intact mitochondria was 4 nmol/min per mg. On exposure of mitochondria to increasing concentrations of digitonin, this activity was fully inhibited, while the CPT_i activity was exposed; the maximal specific activity of the latter attained with optimal, below-inhibitory, concentrations of digitonin was about 7.6 nmol/min per mg (Fig. 1). The possibility that this latter value included any activation of CPT_i by digitonin is eliminated insofar as, unlike other detergents, digitonin did not show (Table 1) such an effect on the CPT_i activity of isolated IMV (where the enzyme activity is not latent) or on the activity of the soluble fraction. (*b*) Under the assay conditions used here, the maximal increase in assayable CPT activity obtained on optimal sonication (by varying the sonication period) of mitochondria was about 2.5-fold. This gives a ratio of 1:1.5 for the two activities. (*c*) During isolation of mitochondrial membranes, a part of the total CPT activity appears in the soluble fraction. Monitoring of these activities in the forward direction together with assays of membrane marker enzymes showed that about 90% of the initially overt CPT activity of mitochondria (5.5 nmol/min per mg), which was malonyl-CoA-sensitive, could be ascribed to the CPT_o of the outer membrane. When the CPT activity of IMV (21.5 nmol/min per mg) was extrapolated to mitochondria, the ratio of CPT_o to CPT_i activity obtained was about 1:1. This changed to a ratio of about 1:1.5 when the CPT activity that became solubilized during the preparation of IMV was assumed to have arisen from the CPT_i itself.

When the CPT activity was monitored as the conversion of palmitoylcarnitine into palmitoyl-CoA, the ratio of CPT_o to CPT_i activities proved much higher, as the following shows. For the backward reaction the specific activity of CPT_o of isolated OMV was 36 nmol/min per mg of OMV protein. On the basis of the OMV protein representing 5% (Tzagoloff, 1982; Wojtczak & Sottocasa, 1972) of the total mitochondrial protein, the calculated value for intact mitochondrial CPT_o was 1.8 nmol/min per mg of mitochondrial protein in the backward assay. The CPT activities recovered in the IMV plus the soluble fractions, when extrapolated to intact mitochondria, on the basis of IMV proteins accounting for 21% (Tzagoloff, 1982) of total mitochondrial protein, and assuming that the soluble CPT activity was derived from CPT_i, gave a value of 78.4 nmol/min per mg of mitochondrial protein. Thus, when measured in the backward direction, the CPT_o to CPT_i activity ratio seemed about 1:40; this high ratio stemmed from the ability of CPT_i to catalyse the backward reaction much faster than the forward reaction as described above.

Phospholipid requirement for the malonyl-CoA inhibition of CPT_o activity

The relationship of the inhibitory, malonyl-CoA-binding, site to the catalytic site of CPT_o is not clear, apart from the information that the two are separate (for review, see Saggerson, 1986; Murthy & Pande, 1987). Regardless of the nature of their interaction, two sets of our observations have indicated that lipids play a role in this process. Thus Table 4 shows that, when exposure of OMV to octyl glucoside led to the solubilization of CPT_o, the CPT_o activity and its malonyl-CoA-inhibitability were both decreased. On subsequent reconstitution into asolectin liposomes, the CPT_o activity was not noticeably affected, but the malonyl-CoA inhibitability was increased (from 49 to 70%). The demon-

Table 4. Effects of octyl glucoside, phospholipase C and reconstitution into liposomes on the CPT_o activity and its inhibition by malonyl-CoA

For octyl glucoside treatment, OMV were incubated with 40 mM-octyl glucoside, at protein/octyl glucoside ratio of 1:10 (w/w) for 45 min at 0 °C in a medium of 100 mM-potassium phosphate, pH 7.4, 20% (v/v) glycerol, 1 mM-EDTA and 1 mM-dithiothreitol. The details of phospholipase C treatment were as described in the Materials and methods section, except that 0.01 unit of phospholipase C was used per mg of OMV protein. For the reconstitution, OMV preparation, after the treatments where shown, was mixed with asolectin liposomes at a lipid/protein ratio of 40:1 (w/w) and then subjected to rapid freezing, followed by thawing at 30 °C, and sonication for 10 s. CPT assay was with 50 μM-palmitoyl-CoA without or with 200 μM-malonyl-CoA. Results are means ± S.E.M. for seven to ten observations: $*P < 0.01$ compared with 'None'; $\dagger P < 0.05$ compared with the octyl glucoside group; $\ddagger P < 0.01$ compared with the phospholipase C group.

Treatment of OMV	CPT_o activity (nmol/min per mg of protein) Without malonyl-CoA	With malonyl-CoA	Inhibition (%)
None	108 ± 6.4	7 ± 1.1	93
None + reconstitution	102 ± 5.9	5 ± 0.5	95
Octyl glucoside	58 ± 5.4*	29 ± 2.5*	49
Octyl glucoside + reconstitution	60 ± 5.5*	18 ± 2.5*†	70
Phospholipase C	101 ± 7.1	58 ± 3.4*	43
Phospholipase C + reconstitution	102 ± 6.5	19 ± 1.6*‡	81

stration of the latter effect required inclusion of a freeze–thaw/sonication step as described in Table 4; a mere provision of lipids in the assay mixture otherwise had little effect on malonyl-CoA sensitivity. Table 4 shows also that, although limited exposure of OMV to phospholipase C had no effect on their CPT_o activity, the ability of malonyl-CoA to inhibit the CPT activity was decreased from 93 to 43%, and on subsequent reconstitution a near-complete restoration of malonyl-CoA-inhibition was observed. It is known that the malonyl-CoA-inhibitability of CPT_o in intact mitochondria is considerably blunted on starvation (for reviews see Bremer, 1983; Hoppel & Brady, 1985). We find that this effect is demonstrable also with isolated OMV. These observations indicate that the regulation of malonyl-CoA-sensitivity of CPT_o eventually involves stable changes in the outer membrane components and raises the possibility of changes in membrane lipids playing a role in this regulation. The exact nature of these changes, however, remains to be defined.

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